Conditioning cognitive abilities in school children: Effect of interventions in the natural environment on salivary neurotransmitters

Synopsis

Submitted in the partial fulfilment for the requirement of

Doctor of Philosophy

in

Zoology

Submitted by-
D. Abhilasha

Under the supervision of

Dr. T. K. Rao
Co-supervisor

Prof. Shabad Preet
Supervisor

Department of Zoology, Faculty of Science

Dayalbagh Educational Institute (Deemed to be University)

Dayalbagh, Agra, 282005

2023

TABLE OF CONTENT

S.No.	TITLE	PAGE NUMBER
1.	Introduction	**1-5**
2.	Literature Review	**5-10**
3.	Objectives	**10**
4.	Materials and Methods	**10-14**
5.	Plan of Work	**14**
6.	References	**15-21**
7.	Appendix	**22**

1. INTRODUCTION:

The evolutionary development of the brain is associated with changes in gross anatomical structures **(Tan et al., 1999)**. However, more recently, the regulation of neurotransmitters **(Saniotis et al., 2020; Zhong et al., 2021)** has been shown to have an impact on cognitive abilities. This paradigm shift unfolds the understanding that provides insight to find (i) neurotransmitters and their ratios as indicator biomarkers in pathophysiological diseases, and (ii) the effect of regulation of neurotransmitters or neuromodulators on cognitive abilities with conditional alterations **(Zhong et al., 2021)** of interventions with a purpose **(Gallen & D'Esposito, 2019; Papadakis et al., 2022).**

Neurotransmitters affect the limbic system and influence behavioral patterns **(Hensler, 2006)**. For instance, catecholamines (serotonin, dopamine, and norepinephrine) regulate mood and are associated with the depressive attributes of the individual **(Qureshi, 2019)**. The basic organization of the catecholamine-containing neuronal systems and their axonal projections in the brain was initially worked out using classical immuno-fluorescence techniques during the 1960s and 1970s **(Björklund & Dunnett, 2007**). Serotonin has serotonergic neurons mainly situated in dorsal and median raphe nuclei, parts of the brain stem. The cellular bodies of these neurons are concentrated at raphe nuclei and their axonal portions extend to the whole brain i.e. frontal lobe, amygdala, hippocampus, striatum, prefrontal cortex, and medial septum **(Hensler, 2006).** Along with serotonin, dopamine is released from dopaminergic neurons mapped mainly at the substantia nigra (SN) and ventral tegmentum area (VTA) **(Radwan et al., 2019).**

The lower levels of dopamine and serotonin are associated with schizophrenia, depression, and Parkinson's diseases indicating the cause of impairment of cognitive functions owing to the lack of dopamine and serotonin. On the contrary, the replenishment of these neurotransmitters or more recently the transplant of dopaminergic neurons in patients suffering from Parkinson's enhances cognition **(Barbuti et al., 2021; Shohamy et al., 2005).** Positron emission tomography (PET) and single-photon emission computed tomography (SPECT) molecular imaging enabled the assessment of brain neurochemical systems by specific biochemical markers of neuroreceptors, transporters, or enzymes **(Cropley et al., 2006)**. Further, versatile immunohistochemical methods, along with a range of highly

sensitive tract-tracing techniques, furnishes a detailed account of the dopamine system elucidated in the brain **(Björklund & Dunnett, 2007**).

The mesencephalon contains two major dopamine (DA) neuron subtypes: the nigral A9 neurons projecting to the striatum along the nigrostriatal pathway and the A10 neurons of the VTA projecting to limbic and cortical areas along mesolimbic and mesocortical pathways. There are 15 main receptors of 5- HT which are further categorized into 7 subfamilies. 5-HT1A serves as an agonist, 5HT2A as the antagonist, 5-HT4 as an agonist, and 5-HT6 as an antagonist for the improvement in cognitive functions in patients with schizophrenia **(Roth et al., 2004).** An increase in attention set-shifting, coordination, reversal learning, and cognitive flexibility are observed with the rise in the levels of adrenaline, dopamine, serotonin, and acetylcholine **(Logue & Gould, 2014)**.

Initially, it was thought that brain size influences intelligence and other cognitive abilities. The evolution of the human brain resolved this controversy as it decreased by about 10% size and had more cranial capacity. The chemical modulation of the brain has shown that the neurotransmitters and neuroendocrine system offer a preferred alternative for correlating cognitive abilities, rather than brain size **(Saniotis et al., 2020).** The authors have demonstrated the altered dopamine levels by the activation of the 5-HTA1 and 5-HT4 receptor in the prefrontal cortex and hippocampus and associated with short-term working memory formation. The change in size and shape of dendritic spines present in the brain is also responsible for the change in working memory **(Gonzalezburgos & Feriavelasco, 2008).** Tryptophan the precursor of serotonin and melatonin regulates the circadian rhythm of the human body. Food rich in tryptophan resulted in an increase in total time in bed, sleep time, sleep efficiency, and immobile period which directed to a better sleep-wake cycle **(Bravo et al., 2013).** Tryptophan levels are also affected by the type of microbiota present in the gut which certainly influences the levels of serotonin in the body. Gut-brain axis influences mood, episodic and working memory, positivity, verbal reasoning, and happiness. Learning with a happy state of mind results in long-lasting memory related to the serotonin levels in the body **(Jenkins et al., 2016).** The concentration of melatonin increases with the decrease in light intensity hence resulting in long-term retention of the memory but does not help in the new memory formation **(Jilg et al., 2019; Zhang et al., 2021).** Cognition is the higher-order functioning of the brain manifested in the form of behavior and actions. These functions involve, for instance,

knowledge acquisition, comprehension, perception, imagination, critical thinking, and analysis, but are not limited to these only **(Pressing & Jolley-Rogers, 1997).** The cognitive ability of any individual can be targeted by changing the pattern of delivery of the information. In recent years some activity-based neurocognitive teaching-learning strategies have been introduced called Brain-based learning strategies **(Ramachandran, 2015)**. Brain-based learning strategies like simulation training, multiple sensory strategies and cooperative learning strategies, bite-sized learning, and active participation with art integrated activity-based methods of learning enhance the learning outcomes in children and lead to knowledge retention **(Gülpinar, 2005)**. These activities are closely associated with cognitive practices and in turn lead to higher memory in children **(Duman, 2010)**. Bloom in 1956 gave 3 discrete domains of human learning namely cognitive, affective and psychomotor. These three domains were further divided into 6 systematic and progressive levels each describing some educational objectives where the accomplishment of one step would lead to the next step. The cognitive domain refers to knowing, or head, affective is emotions or heart, and psychomotor is skill-based learning or hands.

Anderson 2001 reconstructed these domains of learning and gave the basic 4 levels of knowledge which are factual knowledge, conceptual knowledge, procedural knowledge, and metacognitive knowledge. Factual knowledge relates to the collection of information, conceptual knowledge is the conceptualization of facts; procedural knowledge gives an idea about the specific technique or way of doing any task whereas metacognitive knowledge explains one's self **(Leslie, 2016)**. The children engaged in play and growing activities in nature have exposure to the natural microbiota mainly present in the soil. These activities are specifically framed to see the effect of nature and its interventions to see the psycho-cognitive development in children who were belonging to urban areas. The impact of play and grow activities in nature resulted in improved mood especially control of anger, better immunity, and psycho-socio-cognitive understanding **(Sobko et al., 2020).**

The three key attributes are taken into account in this proposal, firstly, neurotransmitters affect and are affected by environmental interventions provided in natural or in school setups. Secondly, these interventions have been demonstrated to enhance cognition **(Chalki et al., 2019, Sobko et al., 2020).** Thirdly, the approach toward the subjects during sample collection and measurements tends to be entirely non-invasive. To achieve this, the saliva of the subjects was adopted as the preferred

physiological sample. Numerous investigators have demonstrated comparable levels of neurotransmitters and metabolites in saliva with blood plasma and cerebrospinal fluid (CSF). For instance, the measurement of acetylcholine, and amino acid derivatives as a diagnostic non-invasive tool for impairment of cognition in AD patients **(Peña-Bautista et al., 2020).** An association of salivary serotonin with social sharing and happiness exhibit yet another study that correlates salivary determination with cognitive trait-like empathy **(Matsunaga et al., 2017).**

In the present study, we intend to gain insight into the impact of interventions conceptually designed in educational and natural setups based on the four levels of knowledge namely factual, conceptual, procedural, and metacognitive knowledge (Anderson and Krathwohl, 2001). The metacognitive intervention includes a blend of natural requisite evolved activities, imperative for the development of cognition.

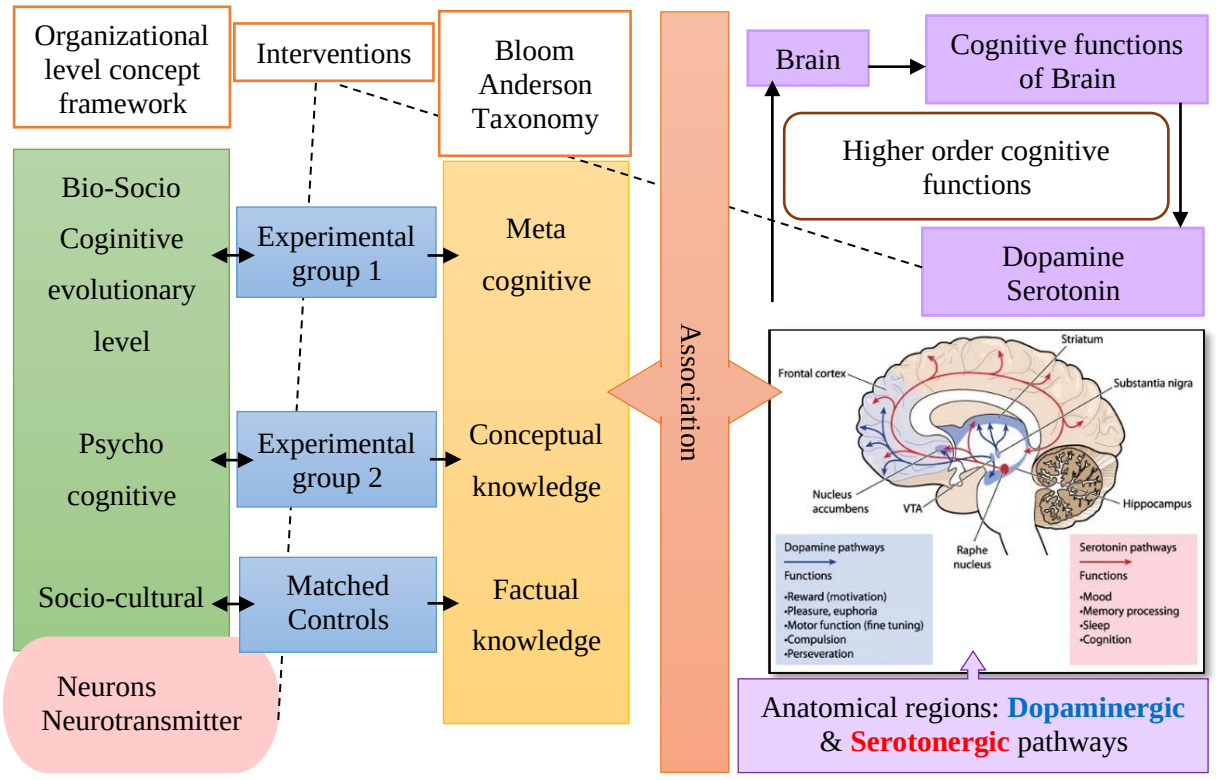

Figure no 1: A concept map showing the potential association of educational (Bloom Anderson Taxonomy) and neurophysiological understanding of cognition. The serotonergic (red) and dopaminergic (blue) neurons have different points of origin in the brain with the defined distribution.

These neuromodulators/neurotransmitters are deemed to affect the cognition of individuals at Psycho-cognitive, Socio-Cultural and Bio-Socio cognitive evolutionary levels. This study targets to bridge the

gap between psychological and biological understanding of cognition. We propose a longitudinal study with long-term exposure to interventions that affect the cognition/ memory of the subjects. We believe that based on psycho-cognitive and neurotransmitter analyses; a prognostic frame of reference will present a foundation for establishing focused custom-made interventions to enhance cognition.

2. LITERATURE REVIEW

The study converges the biological and educational basis of cognition and learning. It aims to establish the effect of activities designed with educational (Boom's and Anderson's taxonomy) and natural setups (Bio-socio cognitive interventions). We target to determine serotonin, dopamine, and melatonin which regulate the higher executive functions like working memory, reward learning, circadian cycle, and emotions like empathy. The review of literature thus has two sections (i) Neurotransmitters: serotonin, dopamine, and melatonin, and (ii) Interventions provided in a specific environment.

(i) Neurotransmitters: A study on brain monoamine neurotransmitters serotonin, dopamine and norepinephrine was conducted to explain the behavior of fish in agonistic and stress states. They also tried to compare the fish behavior with vertebrates and their evolution pattern for the release of neurotransmitters **(Winberg & Nilsson, 1993).** In 2000, Robert M.A. Hirschfeld reviewed the history and evolution of the monoamine hypothesis **(Hirschfeld, 2000)** and in the same year, Pedro L. Delgado gave a review on the lowering of the levels of monoamines in synaptic cleft which was the cause of depression **(Delgado, 2000)**.

Serotonin (5-HT): While studying the patients with major psychological problems and depression, it was observed that they prefer to eat potatoes and other carbohydrate-rich food to enhance their happiness which was brain craving for serotonin, and hence serotoninergic drugs were preferred to control obesity and depression **(Rofey et al., 2012).** Serotonergic neurons have their axonic terminals distributed in the brain stem and spinal cord which project to the muscles of the jaw, face, and neck. Reduced motor activity was observed with a decrease in serotonin levels **(Jacobs & Fornal, 1997)**. The spectrum of behaviors influenced by serotonin such as aggression, anxiety, cardiovascular regulations, pan sensitivity, and learning was studied further **(Lucki, 1998)**. Serotonin has an inhibitory role in aggression and depression and the depletion of serotonin from the prefrontal cortex affects the reversal of learning through stimulus and reward but not the attention set-shifting **(Clarke, 2005).** The inhibition

of serotonin was associated with negative mood and hence correlated with anxiety and depression **(Dayan & Huys, 2008).** There are two serotonergic pathways followed in the brain one arising from median raphe nuclei, and another from dorsal raphe nuclei. The pathway from dorsal raphe nuclei innervates to the amygdala, lateral hippocampus, prefrontal cortex, striatum, and lateral septum. A pathway from ventral raphe nuclei innervates to the dorsal hippocampus, medial septum, and hypothalamus. **(Hensler, 2006).** It was observed that the quantity of serotonin in the extracellular matrix decides the production of serotonin and tryptophan in the brain matrix **(Charnay, 2010)**.

Targeting the whole brain inputs which regulate the serotonergic neurons from dorsal and median raphe emphasizes the importance of hyper direct pathways from the prefrontal cortex, thalamus, hypothalamus, striatum, globus pallidus externa, substantia nigra, and ventral tegmental area **(Pollak Dorocic et al., 2014).** Jenkins 2016 worked on the influence of tryptophan and serotonin on mood and cognition with a possible role in the gut-brain axis. The lower levels of serotonin were associated with deficits in working and episodic memory and verbal reasoning whereas tryptophan supplementation results in positive effects on attention and memory **(Jenkins et al., 2016).** The serotonergic neurons are mainly located in dorsal and ventral raphe nuclei. There are 15 main receptors of 5- HT which are further categorized into 7 subfamilies. 5-HT1A serves as an agonist, 5HT2A as an antagonist, 5-HT4 as an agonist, and 5-HT6 as an antagonist for the improvement of cognitive functions in patients with schizophrenia and other physiological activities in the body **(Roth et al., 2004, Mück-Šeler, 2016)**.

The association of salivary serotonin with social sharing of happiness and empathetic abilities was studied and a negative correlation between the two was observed **(Matsunaga et al., 2017).** Researchers connected the changing patterns of neuromodulators such as serotonin (5-HT) and neurotransmitters like dopamine and nor-epinephrine with emotions. The basic patterns that were studied are punishment, reward and stress. They gave 3 primary color model describing these 3 neurotransmitters and said there are other hormones as well that act in the secondary pathways to modulate the emotional response **(Wang et al., 2020).**

Dopamine (DA): The circuit of mesencephalon neurons localized in basal ganglia, cerebral cortex, and the limbic system was mapped to see the organization and activation of its receptors and their activation in motor behavior, cognitive functions, and reward mechanisms **(Bentivoglio & Morelli, 2005)**. DA

alterations on D2 receptors and a variety of cognitive tasks might be related to motor performance as well as inhibition of competing responses, temporal processing, and sequential organization of behavior **(Cropley et al., 2006).** A review on 2 major classes of dopaminergic receptors D1 like (D1, D5) and D2 like (D2, D3, and D4) was conducted which are G- Protein-coupled receptors (GPCR). The depletion of monoamine levels i.e. the levels of serotonin, dopamine, and norepinephrine leads to mood disorders **(Ruhé et al., 2007)**.

High-performance liquid chromatography (HPLC)-mass spectrometry (MS) was used to test xenobiotic neurotoxicity on chromaffin cells and its effect on the production of epinephrine, norepinephrine, dopamine, and 5-hydroxytryptamine concentrations in the gut **(Carrera et al., 2007)**. Another study by Björklund & Dunnett attempts to summarize the current knowledge of the diversity and neurochemical features of the nine dopamine-containing neuronal cell groups in the mammalian brain, their distinctive cellular properties, and their ability to regulate their dopaminergic transmitter machinery in response to altered functional demands and aging **(Björklund & Dunnett, 2007)**. Hugo Lövheim 2012 has given a new 3D model which explains the emotions i.e. distress, anger, rage, excitement, interest, surprise, enjoyment, fear, and distress which are regulated by different amounts of monoamine neurotransmitters in our body **(Lövheim, 2012).**

Another study concludes the activation of serotonin and dopamine results in the production of another hormone oxytocin which reduce pain perception and increase emotional connection **(Baixauli, 2017).** Optogenetic studies on rodents suggest that amount of 5- HT affects the glutamate concentration in the brain which in turn alters the dopamine levels by changing in reward pattern **(Fischer & Ullsperger, 2017).** The formation of declarative memory by reconsideration of facts or activities is due to the modulation of dopamine in the substantia nigra and hippocampus **(Kamiński et al., 2018).** Dopamine is primarily produced in the substantia nigra (SN) and the ventral tegmentum area (VTA) in the brain. It plays a well-established role in motor control, reward, mood regulation, and addictive behavior. Dopamine release is regulated by the circadian clock and hence, plays a regulatory role in the sleep-wake cycle **(Radwan et al., 2019).**

Studies suggested that obese and diabetic patients are more prone to develop mood disorders due to insulin imbalance and dopamine reduction in the body system **(Kleinridders & Pothos, 2019).** A

comparative study between dopamine in the central and peripheral nervous system summarized that dopamine is a neurotransmitter in the central nervous system associated with reward salience and food intake, whereas peripheral dopamine is involved in sympathetic stress regulation, digestion, and gastrointestinal motility **(Mills et al., 2020).** Neurotransmitters such as serotonin, dopamine, and dihydroxyphenylalanine (DOPA) levels in peripheral blood are related to their levels in cerebrospinal fluid and were found to get altered with the alteration in the conditions of hypoxia in rats **(Zhong et al., 2021).**

Melatonin: Melatonin a derivative of serotonin is secreted from the pineal gland which is known as the alarm clock of our body. Melatonin function indicates to have a role in the biological regulation of circadian rhythms, sleep, mood, and perhaps reproduction, tumor growth, and aging **(Brzezinski, 1997).** The role of melatonin is associated with the priming of sleep-associated brain activation patterns in anticipation of sleep and fatigue (**Gorfine et al., 2006)**. Melatonin can cross the blood-brain barrier and may help in curing perinatal brain injuries in newborn children and thus help in curing learning disabilities **(Bouslama et al., 2007).** Reactive oxygen species influence the neurogenesis of the hippocampus and trigger apoptosis, inflammation, and cell cycle perturbation. It is observed that antioxidants like melatonin and its derivatives may regenerate hippocampal neurogenesis and safeguard cognitive health **(Manda & Reiter, 2010)**.

Tryptophan the precursor of serotonin and melatonin regulates the circadian rhythm of the human body. The concentration of tryptophan can be modulated by including cereal-enriched food and it can reduce depression by enhancing positivity in the elderly population **(Bravo et al., 2013).** The result of over-exercise with sleep deprivation causes a reduction in the activity of melatonin leading to the late onset of the sleep cycle leading to compromised cognitive functioning **(Davis et al., 2014)**. Short-term and long-term memory formation gets impaired by the oxidative stress of the hippocampus caused due to overeating high-fat food. Melatonin treatment prevents such effects **(Alzoubi et al., 2018)**. It has a time-dependent onset of hippocampal signaling and gene expression which improves the learning skills during the daytime **(Jilg et al., 2019).**

(ii) **Interventions provided in a specific environment: -** Neurocognitive model of learning is a constructive approach to learning which ensures the addition of new knowledge to the old acquired

knowledge. Neural networks help to connect the different areas and domains of the brain which enables higher-order thinking **(Anderson, 1992).** The basic principles of brain-based learning and constructivist models of educational learning involve experiential learning, problem-based learning, or cooperative learning which influence the cognitive development of children **(Gülpinar, 2005**). This involves counseling practices for adolescents to manage their moods and emotions and get along with the educational activities **(Langelier & Connell, 2005)**.

The circadian cycle which is the day-night cycle also influences human cognition relating it to attention capacity, executive functioning, and memory **(Schmidt et al., 2007).** Stress and stress hormones also hurt cognition **(Lupien et al., 2007)**. In 2008, the principles of learning and their implications in teaching in a neurocognitive manner were explained **(Goswami, 2008)**. A meta-analysis on the effect of learning skill interventions on student learning was done which proposed that the SOLO (Structure of the observed learning outcome) model of learning should be promoted by involving various cognitive, metacognitive, or affective interventions **(Hattie et al., 1996).** There was a positive influence of physical activities on cognition in the children and hence it was proposed that time for physical activities should be increased in schools **(Fisher et al., 2011).** Bloom in 1965 classified thinking into six cognitive levels of complexity where the lowest levels are knowledge, comprehension, and application, and the highest levels are analysis, synthesis, and evaluation. Anderson and Krathwohl 2001 formulated blooms taxonomy by changing the highest levels of the cognitive domain as evaluation and creation and introduced four levels of knowledge i.e. factual knowledge involving introduction to facts, conceptual knowledge involving combining facts to develop a concept, procedural knowledge involving learning through a specific procedure or skill-based learning and metacognitive knowledge involving knowledge of once own self **(Leslie, 2016).**

Individuals from different patterns remember different things that may vary from person to person **(Logie, 2018)**. Several educational models and games were designed to help children learn the concepts by pay way method using the basis of Bloom's taxonomy and the children were monitored by electroencephalogram (EEG). The wave pattern observed was correlated with the child's output of learning **(Chalki et al., 2019).** A recent article explains how our perception defines cognition which is continuously changing with the change in generations **(Overmann, 2021).**

A study was conducted to see the possible relationship between the fecal serotonin, gut microbiota, and psychosocial behavior of children. They engaged the children in Play and grow activities which were designed to involve them in nature and give them exposure to the natural microbiota mainly present in the soil. These activities were explicitly framed to see the effect of nature and its interventions to see the psycho-cognitive development in children who were belonging to the urban areas. They observed that play and growth activities in nature resulted in improved mood, especially anger, immunity, and psycho-socio-cognitive understanding **(Sobko et al., 2020).**

Therefore, we aim to investigate the impact of interventions provided in the specific environment on the alterations of salivary neurotransmitters that reflects the physiological state of the subjects. This study visions to contribute to designing need-oriented specific interventions based on neurophysiological response toward the enhancement of bio-socio cognitive skills, behavioral control, emotional stability, and merging into neuro-ethics.

3. OBJECTIVES

The study involves 3 neurotransmitters responsible for the cognitive functions of the brain. The objectives targets to translate neurophysiology into cognitive skills with conceptually designed interventions. The experimental groups elaborated in the methods section focused on activities that are (i) interventions given in a natural environment that aims for bio-socio cognitive skills and (ii) interventions given in a school environment that aims for psycho-cognitive skills.

I. Relating levels of salivary serotonin into education and cognition.
II. Relating levels of salivary dopamine into education and cognition.
III. Relating levels of salivary melatonin into education and cognition
IV. To compare the quantitative and qualitative outcomes of psychoanalysis and neurotransmitter levels.

4. MATERIALS AND METHOD

4.1 Materials: The samples will be analyzed for the presence of neurotransmitters in saliva by using the following kits from Elabscience www.elabscience.com.

i) ST/5-HT (serotonin/5-Hydroxytryptamine) ELISA kit, catalog no: E-EL-0033
ii) DA(Dopamine) ELISA kit, catalog no: E-EL-0046
iii) Human MT(Melatonin) ELISA kit, catalog no: E-EL-H2016

Equipment: Biotek Multi-Mode Reader (Synergy H1 Hybrid)

Software for activity-based learning: A computer software specifically designed to target baseline behaviours such as memory, attention, problem solving etc., of the subjects belonging to 6-10 years of age will be used for learning purpose and its effect will be noted down with the time.

Selection criteria of the subjects: Children in the age group 6-10 years are to be considered as the subjects for the study. Grouping of subjects is done based on their exposure to the interventions framed as per Bloom- Anderson's taxonomy of educational objectives.

A. Children who are exposed to meta-cognitive practices in the natural surroundings following activity-based interventions are to be kept under the experimental group 1.
B. Children who are not exposed to any such interventions but go to school to receive factual knowledge are to be kept under the positive control group.
C. Another group like control from Bahadurpur village or from OPD who do not even attend the school are to be kept under the negative control group.

4.2 Methods-

Approval from Ethical Committee: Approval has been taken from the Institutional ethical committee having the code as DEI-IEC/03/01/2021-2022.

Consent form: A consent form will be out filled by the participants or their parents before the collection of saliva. Consent is attached as an appendix.

Sample Size: Approximately 100 samples would be considered for experimenting on repetitive bases including the control and experimental group.

Collection of Sample: Saliva is a biofluid having a composition of 90% of water and the remaining 10% is having numerous constituents (proteins, amino acids, electrolytes, enzymes, immunoglobulin, and neurotransmitters). Saliva is considered an alternative biofluid for blood, CSF, and tissues **(Peña-Bautista et al., 2020)**. It is the noninvasive method for the collection of the sample for various tests but requires various precautions during the sampling and storage **(Zimmermann, 2008).** Saliva samples are to be collected following the Salimetrics protocol in the 50ml centrifuge tube in an icebox maintained at 4 ºC.

Processing and storage of saliva sample: Centrifugation**:** The collected saliva will be transferred and centrifugation of the salivary samples will be performed at 5,500 rpm, 2600g **(Zimmermann, 2008)** to separate the cells and the other constituents from the supernatant. The supernatant will be transferred samples will be stored at -20 ºC for further use. Following precautions are to be taken while collecting the sample.

- **Precautions:**

1. The subjects are supposed not to eat anything before the sampling.
2. The subjects will be given drinking water to drink and rinse their mouth properly.
3. The collection process should not consider any coughing or forceful spitting.

ELISA: Enzyme-linked immunosorbent assay (ELISA) will be done following the standard steps that include **(Shah & Maghsoudlou, 2016).**

1. **Preparation of standard working solutions:** The standards will be prepared by serial dilution of the reference standard and sample diluent to the main stock solution and a standard graph will be generated. The range of standard will be from 1000 ng/ml to 15.63 ng/ml **(Aydin, 2015).**
2. **Preparation of washing solutions:** The concentrated washing buffers will be diluted by adding a sufficient amount of distilled water to them **(Aydin, 2015).**
3. **Biotinylated Detection Ab working solutions:** The working solution of Biotinylated Detection Ab will be produced by adding concentrated Biotinylated Detection Ab to Biotinylated Detection Ab diluent in 1:99 proportions.
4. **HRP conjugate working solution:** The working solution of HRP conjugate will be produced by adding concentrated HRP conjugate to HRP conjugate diluent in 1:99 proportions.

The ELISA test will be performed for serotonin, dopamine, and melatonin to determine their concentrations in saliva in a longitudinal study planned for 2 years.

These determinations will be conducted by collecting fresh samples of saliva at every 6 months gap.

Psychoanalytical Study: A self-constructed psychoanalytical tool/ observational schedule will be developed. The observational schedule is been included in the study to gather the information about the individual participants related to their exposure to schools, natural surroundings, food habits, social

status of their family etc. A pilot study will be conducted to check the validity and reliability and lastly, it will be implicated on the subjects.

Demographic study of the children: The participant information and a consent form will be filled out by the children and their parents. The participant information includes the basic details about the subject and their parents whereas the consent form is to be given after explaining the sampling, objective of sampling, method, etc. The participant will be kept free to choose to give the sample.

Table 1: the below figure shows the information form which will be filled by the participant or their parents.

Participant Information Form	
Name of participant	
ID/ Code	
Gender	
Age	
Class	
Date of Birth	
Under intervention since	
Fathers Name	
Fathers Educational Status	
Fathers Mobile number	
Mothers Name	
Mothers Educational Status	
Mothers Mobile number	
Blood Pressure	
Temperature	

Table 2: The parameters of groups to be considered under the following timeline.

S.No.	**Demographics of all geographical areas**
1	**Parameters:** Age, Gender, Parents Education, Body temperature, Oxygen levels
2	**Groups:** Experimental group1, Experimental group 2, Positive control group, Negative control group (Each group has at least 30 subjects)
3	**Frequency** of sampling for each subject for 3-5 days at the respective time given below in the timeline

Time Line:

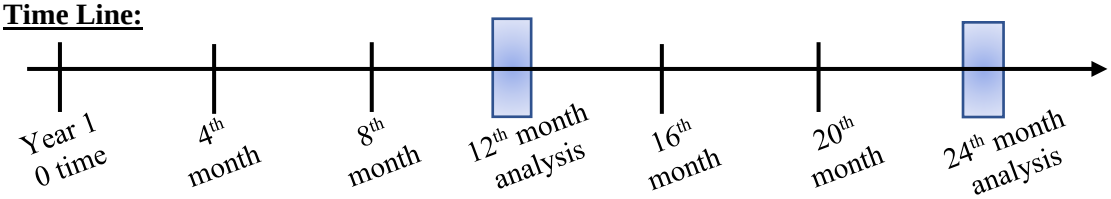

Figure no 2: The timeline showing the frequency of sampling.

Level of neurotransmitters in triplicates ⟶ monitoring variation across days, variation across months final analysis in 2nd year

Statistical Analysis: Statistical analysis will be performed using Student t-test, ANOVA, and chi-square test to compare the experimental group with their matched controls using ORIGIN_Pro software (version- 8.5) (**Matsunaga et al., 2017)**.

The rating scale obtained from the psychological test will be analyzed concerning the experimental results and a correlational analysis will be conducted between cognition with learning methodologies, geographical background, and age. Details have been incorporated in the main synopsis.

5. PLAN OF WORK

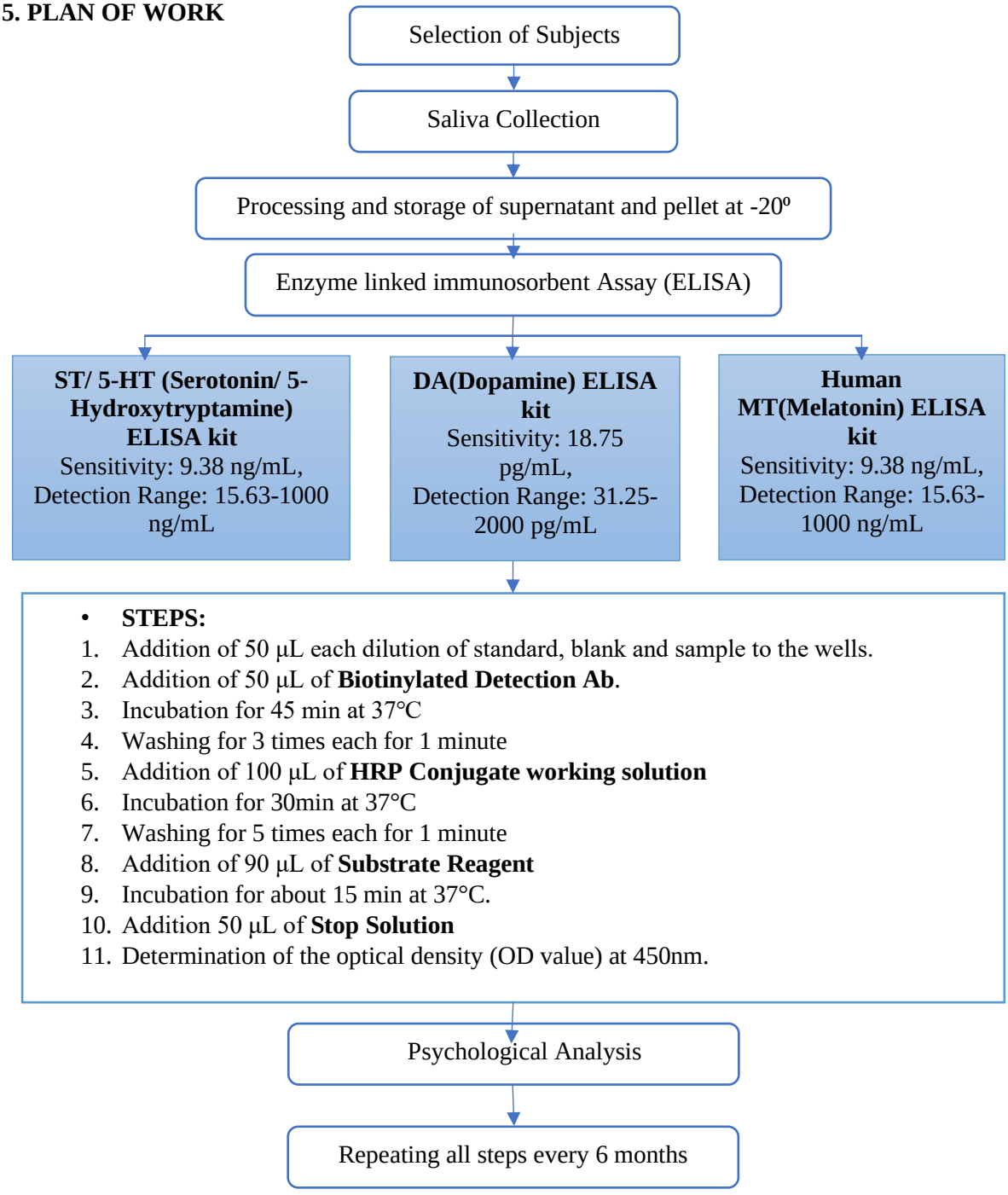
Selection of Subjects
Saliva Collection
Processing and storage of supernatant and pellet at -20º
Enzyme linked immunosorbent Assay (ELISA)
ST/ 5-HT (Serotonin/ 5-Hydroxytryptamine) ELISA kit
Sensitivity: 9.38 ng/mL,
Detection Range: 15.63-1000 ng/mL
DA(Dopamine) ELISA kit
Sensitivity: 18.75 pg/mL,
Detection Range: 31.25-2000 pg/mL
Human MT(Melatonin) ELISA kit
Sensitivity: 9.38 ng/mL,
Detection Range: 15.63-1000 ng/mL
• STEPS:
1. Addition of 50 µL each dilution of standard, blank and sample to the wells.
2. Addition of 50 µL of Biotinylated Detection Ab.
3. Incubation for 45 min at 37°C
4. Washing for 3 times each for 1 minute
5. Addition of 100 µL of HRP Conjugate working solution
6. Incubation for 30min at 37°C
7. Washing for 5 times each for 1 minute
8. Addition of 90 µL of Substrate Reagent
9. Incubation for about 15 min at 37°C.
10. Addition 50 µL of Stop Solution
11. Determination of the optical density (OD value) at 450nm.
Psychological Analysis
Repeating all steps every 6 months

Figure no 3: Plan of work

6. Bibliography

1. Alzoubi, K. H., Mayyas, F. A., Mahafzah, R., & Khabour, O. F. (2018). Melatonin prevents memory impairment induced by high-fat diet: Role of oxidative stress. *Behavioral Brain Research*, *336*, 93–98. https://doi.org/10.1016/j.bbr.2017.08.047
2. Anderson, O. R. (1992). Some interrelationships between constructivist models of learning and current neurobiological theory, with implications for science education. *Journal of Research in Science Teaching*, *29*(10), 1037–1058. https://doi.org/10.1002/tea.3660291004
3. Aydin, S. (2015). A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides*, *72*, 4–15. https://doi.org/10.1016/j.peptides.2015.04.012
4. Baixauli, E. (2017). Happiness: Role of Dopamine and Serotonin on Mood and Negative Emotions. *Emergency Medicine: Open Access*, *07*(02), 2–4. https://doi.org/10.4172/21657548.1000350
5. Barbuti, P. A., Barker, R. A., Brundin, P., Przedborski, S., Papa, S. M., Kalia, L. V., & Mochizuki, H. (2021). Recent Advances in the Development of Stem-Cell-Derived Dopaminergic Neuronal Transplant Therapies for Parkinson's Disease. *Movement Disorders*, *36*(8), 1772–1780. https://doi.org/10.1002/mds.28628
6. Bentivoglio, M., & Morelli, M. (2005). The organization and circuits of mesencephalic dopaminergic neurons and the distribution of dopamine receptors in the brain. *Chemical Neuroanatomy*, *21*.
7. Björklund, A., & Dunnett, S. B. (2007). Dopamine neuron systems in the brain: an update. *Trends in Neurosciences*, *30*(5), 194–202. https://doi.org/10.1016/j.tins.2007.03.006
8. Boparai, J. K., Singh, S., & Kathuria, P. (2018). How to design and validate a questionnaire: a guide. *Current clinical pharmacology*, *13*(4), 210-215.
9. Bouslama, M., Renaud, J., Olivier, P., Fontaine, R. H., Matrot, B., Gressens, P., & Gallego, J. (2007). Melatonin prevents learning disorders in brain-lesioned newborn mice. *Neuroscience*, *150*(3), 712–719. https://doi.org/10.1016/j.neuroscience.2007.09.030
10. Bravo, R., Matito, S., Cubero, J., Paredes, S. D., Franco, L., Rivero, M., Rodríguez, A. B., & Barriga, C. (2013). Tryptophan-enriched cereal intake improves nocturnal sleep, melatonin, serotonin, and

total antioxidant capacity levels and mood in elderly humans. *AGE*, *35*(4), 1277–1285. https://doi.org/10.1007/s11357-012-9419-5

11. Brzezinski, A. (1997). Melatonin in Humans. *New England Journal of Medicine*, *336*(3), 186–195. https://doi.org/10.1056/NEJM199701163360306
12. Carrera, V., Sabater, E., Vilanova, E., & Sogorb, M. A. (2007). A simple and rapid HPLC– MS method for the simultaneous determination of epinephrine, norepinephrine, dopamine, and 5-hydroxytryptamine: Application to the secretion of bovine chromaffin cell cultures. *Journal of Chromatography B*, *847*(2), 88–94. https://doi.org/10.1016/j.jchromb.2006.09.032
13. Chalki, P., Tsiara, A., & Mikropoulos, T. A. (2019). An educational neuroscience approach in the design of digital educational games. *Themes in ELearning*, *12*(12), 17–34.
14. Charnay, Y. (2010). Brain serotonergic circuitries. *Dialogues in Clinical Neuroscience*, *12*(4), 471–487. https://doi.org/10.31887/dcns.2010.12.4/ycharnay
15. Clarke, H. F. (2005). Prefrontal Serotonin Depletion Affects Reversal Learning But Not Attentional Set Shifting. *Journal of Neuroscience*, *25*(2), 532–538. https://doi.org/10.1523/jneurosci.3690-04.2005
16. Cropley, V. L., Fujita, M., Innis, R. B., & Nathan, P. J. (2006). Molecular Imaging of the Dopaminergic System and its Association with Human Cognitive Function. *Biological Psychiatry*, *59*(10), 898–907. https://doi.org/10.1016/j.biopsych.2006.03.004
17. Davis, G. R., Etheredge, C. E., Marcus, L., & Bellar, D. (2014). Prolonged Sleep Deprivation and Continuous Exercise: Effects on Melatonin, Tympanic Temperature, and Cognitive Function. *BioMed Research International*, *2014*, 1–6. https://doi.org/10.1155/2014/781863
18. Dayan, P., & Huys, Q. J. M. (2008). Serotonin, Inhibition, and Negative Mood. *PLoS Computational Biology*, *4*(2), e4. https://doi.org/10.1371/journal.pcbi.0040004
19. Delgado, P. L. (2000). Depression: The case for a monoamine deficiency. *Journal of Clinical Psychiatry*, *61*(SUPPL. 6), 7–11.
20. Duman, B. (2010). The Effects of Brain-Based Learning on the Academic Achievement of Students with Different Learning Styles. *Educational Sciences: Theory & Practice*, *10*(4), 2077–2103.

21. Fischer, A. G., & Ullsperger, M. (2017). An Update on the Role of Serotonin and its Interplay with Dopamine for Reward. *Frontiers in Human Neuroscience*, *11*(October), 1–10. https://doi.org/10.3389/fnhum.2017.00484
22. Fisher, A., Boyle, J. M. E., Paton, J. Y., Tomporowski, P., Watson, C., McColl, J. H., & Reilly, J. J. (2011). Effects of a physical education intervention on cognitive function in young children: randomized controlled pilot study. *BMC Pediatrics*, *11*(1), 97. https://doi.org/10.1186/1471-2431-11-97
23. Gallen, C. L., & D'Esposito, M. (2019). Brain Modularity: A Biomarker of Intervention-related Plasticity. *Trends in Cognitive Sciences*, *23*(4), 293–304. https://doi.org/10.1016/j.tics.2019.01.014
24. Gonzalezburgos, I., & Feriavelasco, A. (2008). Serotonin/dopamine interaction in memory formation. In *Serotonin– Dopamine Interaction: Experimental Evidence and Therapeutic Relevance* (Vol. 172, Issue 08, pp. 603–623). Elsevier. https://doi.org/10.1016/S0079-6123(08)00928-X
25. Gorfine, T., Assaf, Y., Goshen-Gottstein, Y., Yeshurun, Y., & Zisapel, N. (2006). Sleepanticipating effects of melatonin in the human brain. *NeuroImage*, *31*(1), 410–418. https://doi.org/10.1016/j.neuroimage.2005.11.024
26. Goswami, U. (2008). Principles of Learning, Implications for Teaching: A Cognitive Neuroscience Perspective. *Journal of Philosophy of Education*, *42*(3–4), 381–399. https://doi.org/10.1111/j.1467-9752.2008.00639.x
27. Gülpinar, M. A. (2005). The Principles of Brain-Based Learning and Constructivist Models in Education. *5*(November 2005), 299–306.
28. Hattie, J., Biggs, J., & Purdie, N. (1996). Effects of Learning Skills Interventions on Student Learning: A Meta-Analysis. *Review of Educational Research*, *66*(2), 99–136. https://doi.org/10.3102/00346543066002099
29. Hensler, J. G. (2006). Serotonergic modulation of the limbic system. *Neuroscience & Biobehavioral Reviews*, *30*(2), 203–214. https://doi.org/10.1016/j.neubiorev.2005.06.007
30. Hirschfeld, R. M. A. (2000). History and evolution of the monoamine hypothesis of depression. *Journal of Clinical Psychiatry*, *61*(SUPPL. 6), 4–6.

31. Jacobs, B. L., & Fornal, C. A. (1997). Serotonin and motor activity. *Current Opinion in Neurobiology*, *7*(6), 820–825. https://doi.org/10.1016/S0959-4388(97)80141-9
32. Jenkins, T., Nguyen, J., Polglaze, K., & Bertrand, P. (2016). Influence of Tryptophan and Serotonin on Mood and Cognition with a Possible Role of the Gut-Brain Axis. *Nutrients*, *8*(1), 56. https://doi.org/10.3390/nu8010056
33. Jilg, A., Bechstein, P., Saade, A., Dick, M., Li, T. X., Tosini, G., Rami, A., Zemmar, A., & Stehle, J. H. (2019). Melatonin modulates daytime-dependent synaptic plasticity and learning efficiency. *Journal of Pineal Research*, *66*(3). https://doi.org/10.1111/jpi.12553
34. Kamiński, J., Mamelak, A. N., Birch, K., Mosher, C. P., Tagliati, M., & Rutishauser, U. (2018). Novelty-Sensitive Dopaminergic Neurons in the Human Substantia Nigra Predict Success of Declarative Memory Formation. *Current Biology*, *28*(9), 1333-1343.e4. https://doi.org/10.1016/j.cub.2018.03.024
35. Kleinridders, A., & Pothos, E. N. (2019). Impact of Brain Insulin Signaling on Dopamine Function, Food Intake, Reward, and Emotional Behavior. *Current Nutrition Reports*, *8*(2), 83–91. https://doi.org/10.1007/s13668-019-0276-z
36. Langelier, C. A., & Connell, J. D. (2005). Emotions and learning: Where brain-based research and cognitive-behavioral counseling strategies meet the road. *River College Online Academic Journal*, *1*(1), 1–13.
37. Leslie, C. (2016). Anderson and Krathwohl Bloom's Taxonomy Revised.
38. Logie, R. (2018). Human Cognition : Common Principles and Individual Variation. *Journal of Applied Research in Memory and Cognition*, 1–16. https://doi.org/10.1016/j.jarmac.2018.08.001
39. Logue, S. F., & Gould, T. J. (2014). The neural and genetic basis of executive function: Attention, cognitive flexibility, and response inhibition. *Pharmacology Biochemistry and Behavior*, *123*, 45–54. https://doi.org/10.1016/j.pbb.2013.08.007
40. Lövheim, H. (2012). A new three-dimensional model for emotions and monoamine neurotransmitters. *Medical Hypotheses*, *78*(2), 341–348. https://doi.org/10.1016/j.mehy.2011.11.016

41. Lucki, I. (1998). The spectrum of behaviors is influenced by serotonin. *Biological Psychiatry*, *44*(3), 151–162. https://doi.org/10.1016/S0006-3223(98)00139-5

42. Lupien, S. J., Maheu, F., Tu, M., Fiocco, A., & Schramek, T. E. (2007). The effects of stress and stress hormones on human cognition: Implications for the field of brain and cognition. *Brain and Cognition*, *65*(3), 209–237. https://doi.org/10.1016/j.bandc.2007.02.007

43. Manda, K., & Reiter, R. J. (2010). Melatonin maintains adult hippocampal neurogenesis and cognitive functions after irradiation. *Progress in Neurobiology*, *90*(1), 60–68. https://doi.org/10.1016/j.pneurobio.2009.10.019

44. Matsunaga, M., Ishii, K., Ohtsubo, Y., Noguchi, Y., Ochi, M., & Yamasue, H. (2017). Association between salivary serotonin and the social sharing of happiness. *PLOS ONE*, *12*(7), e0180391. https://doi.org/10.1371/journal.pone.0180391

45. Mills, J. G., Thomas, S. J., Larkin, T. A., & Deng, C. (2020). Overeating and food addiction in Major Depressive Disorder: Links to peripheral dopamine. *Appetite*, *148*, 104586. https://doi.org/10.1016/j.appet.2020.104586

46. Overmann, K. A. (2021). The material difference in human cognition. *Adaptive Behavior*, *29*(2), 123–135. https://doi.org/10.1177/1059712320930738

47. Papadakis, S., Alexandraki, F., & Zaranis, N. (2022). Mobile device use among preschool-aged children in Greece. *Education and Information Technologies*, *27*(2), 2717–2750. https://doi.org/10.1007/s10639-021-10718-6

48. Peña-Bautista, C., Torres-Cuevas, I., Baquero, M., Ferrer, I., García, L., Vento, M., & CháferPericás, C. (2020). Early neurotransmission impairment in non-invasive Alzheimer Disease detection. *Scientific Reports*, *10*(1), 16396. https://doi.org/10.1038/s41598-020-73362-z

49. Pollak Dorocic, I., Fürth, D., Xuan, Y., Johansson, Y., Pozzi, L., Silberberg, G., Carlén, M., & Meletis, K. (2014). A Whole-Brain Atlas of Inputs to Serotonergic Neurons of the Dorsal and Median Raphe Nuclei. *Neuron*, *83*(3), 663–678. https://doi.org/10.1016/j.neuron.2014.07.002

50. Pressing, J., & Jolley-Rogers, G. (1997). Spectral properties of human cognition and skill. *Biological Cybernetics*, *76*(5), 339–347. https://doi.org/10.1007/s004220050347

51. Qureshi, H. J. (2019). Review Article Role of neurotransmitters in the human body (Vol. 01, Issue 03).
52. Radwan, B., Liu, H., & Chaudhury, D. (2019). The role of dopamine in mood disorders and the associated changes in circadian rhythms and sleep-wake cycle. *Brain Research*, *1713*, 42–https://doi.org/10.1016/j.brainres.2018.11.031
53. Ramachandran, S. (2015). Modern Method of Teaching and Learning through Neurocognition : An Innovative Brain Based Strategy for Teachers and Learners. *III*(2), 2141–2151.
54. Rofey, D., Blake, R., & Phillips, J. (2012). Obesity and Depression. In *Cognitive-Behavior Therapy for Children and Adolescents*. American Psychiatric Publishing. https://doi.org/10.1176/appi.books.9781615370955.es11
55. Roth, B., Hanizavareh, S. M., & Blum, A. (2004). Serotonin receptors represent highly favorable molecular targets for cognitive enhancement in schizophrenia and other disorders. *Psychopharmacology*, *174*(1), 17–24. https://doi.org/10.1007/s00213-003-1683-8
56. Ruhé, H. G., Mason, N. S., & Schene, A. H. (2007). Mood is indirectly related to serotonin, norepinephrine and dopamine levels in humans: a meta-analysis of monoamine depletion studies. *Molecular Psychiatry*, *12*(4), 331–359. https://doi.org/10.1038/sj.mp.4001949
57. Saniotis, A., Grantham, J. P., Kumaratilake, J., & Henneberg, M. (2020). Neuro-hormonal Regulation Is a Better Indicator of Human Cognitive Abilities Than Brain Anatomy: The Need for a New Paradigm. *Frontiers in Neuroanatomy*, *13*(January), 1–11. https://doi.org/10.3389/fnana.2019.00101
58. Schmidt, C., Collette, F., Cajochen, C., & Peigneux, P. (2007). A time to think: Circadian rhythms in human cognition. *Cognitive Neuropsychology*, *24*(7), 755–789. https://doi.org/10.1080/02643290701754158
59. Shah, K., & Maghsoudlou, P. (2016). Enzyme-linked immunosorbent assay (ELISA): the basics. *British Journal of Hospital Medicine*, *77*(7), C98–C101. https://doi.org/10.12968/hmed.2016.77.7.C98
60. Shohamy, D., Myers, C. E., Grossman, S., Sage, J., & Gluck, M. A. (2005). The role of dopamine

in cognitive sequence learning: evidence from Parkinson's disease. *Behavioural Brain Research*, *156*(2), 191–199. https://doi.org/10.1016/j.bbr.2004.05.023

61. Sobko, T., Liang, S., Cheng, W. H. G., & Tun, H. M. (2020). Impact of outdoor nature - related activities on gut microbiota, fecal serotonin, and perceived stress in preschool children: the Play & Grow randomized controlled trial. *Scientific Reports*, 1–12. https://doi.org/10.1038/s41598-020-78642-2
62. Švob Štrac, D., Pivac, N., & Mück-Šeler, D. (2016). The serotonergic system and cognitive function. *Translational Neuroscience*, *7*(1), 35–49. https://doi.org/10.1515/tnsci-2016-0007
63. Tan, Ü., Tan, M., Polat, P., Ceylan, Y., Suma, S., & Okur, A. (1999). Magnetic resonance imaging brain size/IQ relations in Turkish University students. *Intelligence*, *27*(1), 83–92. https://doi.org/10.1016/S0160-2896(99)00015-X
64. Wang, F., Yang, J., Pan, F., Ho, R. C., & Huang, J. H. (2020). Editorial: Neurotransmitters and Emotions. *Frontiers in Psychology*, *11*(January), 10–12. https://doi.org/10.3389/fpsyg.2020.00021
65. Winberg, S., & Nilsson, G. E. (1993). Roles of brain monoamine neurotransmitters in agonistic behaviour and stress reactions, with particular reference to fish. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology, and Endocrinology*, *106*(3), 597–614. https://doi.org/10.1016/0742-8413(93)90216-8
66. Zhang, Z., Adamo, K. B., Ogden, N., Goldfield, G. S., Okely, A. D., Kuzik, N., Crozier, M., Hunter, S., Predy, M., & Carson, V. (2021). Associations between sleep duration, adiposity indicators, and cognitive development in young children. *Sleep Medicine*, *82*, 54–60. https://doi.org/10.1016/j.sleep.2021.03.037
67. Zhong, Z., Zhou, S., Xiang, B., Wu, Y., Xie, J., & Li, P. (2021). Association of Peripheral Plasma Neurotransmitters with Cognitive Performance in Chronic High-altitude Exposure. *Neuroscience*, *463*, 97–107. https://doi.org/10.1016/j.neuroscience.2021.01.031
68. Zimmermann, L. K. (2008). A salivary collection method for young children. *Psychophysiology*, *45*(3), 353–355. https://doi.org/10.1111/j.1469-8986.2008.00647.x

7. Appendix:

Consent to participation in the project: Neurotransmitters and learning

I willingly desire to participate in a research project conducted by Ms. D.Abhilasha and Dr. Shabad Preet from Dayalbagh Educational Institute. I understand that the project is designed to collect information about the academic work of the faculty on campus. I will be one of the approximately 80 students participating in this research.

1. My participation in this project is **voluntary**.
2. I understand that I will not be paid for my participation.
3. I may withdraw from participation at any time without penalty. If I refuse to participate in the study or withdraw my participation, no one on my campus will be informed.
4. I understand that most subjects will find the purpose of this research interesting and worthwhile. However, if I / feel uncomfortable in any way during the process of the session, so I have the right to terminate the response or the study of any question.
5. The participation includes non-invasively giving saliva samples by students in a medical camp organized under the National Social Service Camp. This will last for about 10 minutes. The detailed information in the attached sample will be taken with care during sampling.
6. I understand that the researchers will not use the information obtained from this interview to identify me by name in any report and that my confidentiality as a participant in this study will be protected. Subsequent use of records and data will be subject to standard data use policies that protect the anonymity of individuals and institutions.
7. The faculty and administrators from my campus will not be present at the time of sampling.
8. I understand that this research study has been reviewed and approved by the Institutional Ethical for studying human subjects: **Institutional Ethical Committee at Dayalbagh Educational Institute.**
9. I have read and understood the explanation given by me. All my questions have been answered to my satisfaction and I agree to **voluntarily participate** in this study.
10. I have received a copy of this ICF.

My name/ signature with date _____ Signature of the Investigator _____

For more details please contact: Dr.Shabad Preet, Tel : 8979193337 , shabdpreet@dei.ac.in